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(54) Title: IMMOBILIZATION OF NUCLEIC ACIDS

(57) Abstract

The selective immobilization of a nucleic acid molecule with a restriction enzyme half-site at one end is achieved by annealing this half-site to a complementary half-site of a single or double-stranded oligonucleotide coupler molecule that is bound, preferably covalently, to a solid support.

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IMMOBILIZATION OF NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

This invention relates to methods of immobilizing nucleic acid molecules by binding them to a solid support. In particular, it relates to an intermediate coupling or coupler molecule which maximizes the usual inherent advantages of immobilization. In addition, this invention provides for selective and reversible binding through complementary restriction enzyme half sites, a subset of DNA molecules within a heterogeneous population.

DNA, or deoxyribonucleic acid, is a long-chain polymer which comprises the genetic material of all living cells and of many viruses. It embodies in its structure the information required for the synthesis of protein by the cell. That information is transcribed and translated into protein structure by molecules of another and similar polymer, RNA (ribonucleic acid) which occur in various forms in the cell.

The nucleic acids DNA and RNA are composed of monomeric units called nucleotides. A nucleotide consists of a pentose sugar molecule (in DNA, deoxyribose; in RNA, ribose) attached to a phosphate group, and a nitrogenous heterocyclic base linked to the glycosidic carbon of the sugar. The base characterizes the nucleotide. In DNA there are four bases: adenine ("A"), guanine ("G"), cytosine ("C") and thymine ("T"). The bases in RNA are A, G, C, and uracil ("U").

To form the nucleic acids, nucleotides are connected by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses. The result is a chain from which the bases project perpendicularly outward. The code for protein resides in the sequence of bases in DNA. A particular triplet of DNA bases (or "codon") specifies a particular one of the 20 amino acids which are the normal constituents of protein.

Natural DNA is formed as two anti-parallel strands.

The bases on each strand extend toward those on the other. Hydrogen bonds between pairs of opposed bases hold the two strands together. The base sequences of the two strands are complementary; that is, an A on either strand is always opposed by a T on the other, and a C on either strand by a G on the other. The base on one strand and its complementary opposed base from the other strand is called a base pair; the possible base pairs of DNA are thus AT, TA, CG, and GC. DNA is replicated in nature by the simultaneous synthesis of complementary DNA on both original strands.

In RNA the thymine base is replaced by uracil ("U"), but the same complementarity between bases exists. When a cell is to synthesize a particular protein coded on a region of its DNA, it begins by synthesizing an RNA strand complementary to that region. This process, called "transcription", takes place at the DNA molecule, which is used as a template. The synthesized messenger RNA ("mRNA") then becomes a kind of template for "translation", i.e., the synthesis of protein according to the code carried by the mRNA.

It is of great importance to be able to manipulate sequences of nucleic acids and polynucleotides, so as to ascertain and to alter the sequences in vivo and in vitro. The methods of manipulation of nucleic acids include treatment with enzymes and with less complex chemicals, biological manipulations in which living cells are employed, and physical manipulations.

In nature as well as in the laboratory, enzymes -- proteins which catalyze specific biochemical reactions -- are used to form or break bonds in nucleotides. Many of these enzymes have been derived from bacteria or from bacteriophages (bacterial viruses). Among the most useful enzymes in modern genetic engineering are the ligases, which join polynucleotides end-to-end, and the restriction endonucleases, which cleave them (Roberts, R.J., CRC Crit. Rev. Biochem. 1, 123 (1976); Fuchs,

R. and Blakesley, R., Methods in Enzymology 100, 3 (1983)). A restriction endonuclease recognizes a specific base sequence (called its "recognition site") and cleaves DNA at that site, hydrolyzing phosphodiester bonds on both strands. Many restriction endonucleases cleave the two strands at bonds removed by a few nucleotides from each other, thus producing short single stranded regions at each of the cleaved ends. These self-complementary ends, now called half-sites, are then cohesive and may be re-joined. Since all the cleaved ends produced by that particular restriction endonuclease are identical, heterologous DNA sequences which have been cleaved by the same restriction endonuclease may be joined to each other at their half-sites.

In manipulations of oligonucleotides and nucleic acids, it has often been useful to bind the macromolecules to solid supports. (Gilham, P.T., J. Amer. Chem. Soc. 86, 4982 (1964)). Efforts have generally been of two kinds. One kind of activity has been directed to the synthesis of single-stranded polynucleotides by attaching one nucleotide to a support and then adding mono-or oligonucleotides as needed to achieve the desired sequence (Matteucci, M.D. and Caruthers, M.H., Tetrahedron Letters, 21, 719-722 (1980); U.S. Letters Patent No. 4,373,071 to Itakura).

A distinctly different objective is that of binding an already existing polynucleotide to a solid or insoluble support, preferably covalently. This process, which has been called "immobilization", presents many advantages in working with the bound polynucleotide. For example, it can reduce the number of manipulations, facilitate the separation of reaction components, and reduce losses of the polynucleotide or nucleic acid as compared to soluble methods. Immobilized DNA reacts more reliably and reproducibly with enzymes because the attachment density can be controlled and the number of manipulative steps minimized. Finally, immobilized DNA also provides the opportunity for an affinity matrix for nucleic acid enzymes (Alberts, B. M. and

Herrick, G. in Methods of Enzymology XXI, 198-217 (1971)) and other nucleic acids (Shih, T.Y. and Martin, M.A., Biochemistry 13, 3411 (1974)).

There are two ways by which DNA may be bound to a solid support. Most commonly, the binding has been achieved by random coupling along the sugar-phosphate "backbone" of the DNA by covalent (2, 3) and non-covalent (1, 4) methods. (1) Southern, E., J. Mol. Biol. 98, 503 (1975); (2) Litman, R. M., J. Biol. Chem. 243, 6222-6233 (1968); (3) Alwine, J. C., Kemp, D. J. and Stark, G. R., Proc. Natl. Acad. Sci. 74, 5350 (1977); (4) Schabler, H., Nusslein, C. Bonhoeffer, F. J., Kurz, C. and Nietzschmann, I., Eur. J. Biochem. 26, 474-481 (1971). Binding by these methods is unsatisfactory for many applications. For example, because of steric hindrance one or more portions of the immobilized DNA may be relatively inaccessible for manipulation.

A second and preferable way to immobilize DNA strands is by one end. Various techniques have been used (Robberson, D. and Davidson, N., Biochemistry 11, 533-537 (1972); Gilham, P.T., Methods Enzymol. 21, 191-197 (1971); U.S. Letters Patent No. 4,302,204 to Wahl, et al.). Several enzymes have already been used with immobilized DNA, including T4 DNA ligase (Cozzarelli, et al., BBRC 28, 578 (1967)), pancreatic DNase, and E. coli RNA polymerase (Rickwood, D.,BBA 269, 47 (1972)). But even these methods fail to achieve the full potential of immobilization. In some of them, a substantial proportion of the DNA binds to the solid support along its backbone because of secondary reactions between the DNA and the support. Additional shortcomings such as contamination of eluate, high pH coupling which destroys DNA and ultraviolet damage to DNA are known (Potuzak, H. and Dean, P. D. G., FEBS Lett. 88, 161 (1978)). Other disadvantages result from the failure of these methods to provide for specific orientation, that is, binding of all molecules at corresponding ends. Further, once a DNA molecule is covalently immobilized by these methods it cannot ordinarily be easily

released at its point of attachment and recovered intact.

SUMMARY OF THE INVENTION

According to one aspect of our invention, DNA molecules are immobilized selectively, reversibly and specifically, by binding them to a support via restriction endonuclease half-sites. This is achieved by providing a "coupler" molecule, comprising a single or double-stranded oligonucleotide with an endonuclease restriction half site at one end, and covalently attaching that coupler to the solid support at the coupler's other end. The result is a nucleic acid-matrix system utilizing the selective affinity of nucleic acids having complementary restriction half-sites.

According to another aspect of the invention, a coupler is covalently bound to a solid support to form a coupler matrix system. Such a coupler comprises a DNA molecule having at one end a restriction enzyme half site and at its other end a reactive chemical moiety for covalent coupling to a solid support. Such a coupler matrix system can be used to immobilize DNA. The DNA to be immobilized should have a restriction enzyme half site end complementary to that of the coupler. If this DNA does not have the appropriate half-site on its end, it may be cleaved with a suitable restriction enzyme. Further, a desired orientation of the DNA can be achieved by cleavage with different restriction endonucleases, one that generates a half-site complementary to the half site of the coupler. Mixing the DNA and the coupler-matrix followed by incubation with DNA ligase immobilizes the DNA of interest by a covalent bond. DNA can also be attached non-covalently through the terminal restriction site by hydrogen bonding to the terminal restriction site of the coupler matrix.

A further aspect of the invention involves the provision of a coupler bound to DNA. The juncture of the coupler and DNA employs complementary restriction enzyme half sites. These

bound components can be immobilized by covalent reaction of the coupler's other end to a solid support.

Once immobilized in the nucleic acid matrix system, the DNA molecule is freely accessible to a wide variety of uses, for example in hybridization or as a substrate for a number of molecular reactions. There are many advantages of this approach. First, since the DNA is attached at only its end, the great majority of it is free to interact with other nucleic acids or proteins. In addition, the coupler provides a "spacer arm" permitting maximal access to the DNA with minimal steric hindrance from the solid support. Second, because the point of attachment is through a restriction site, a specific rather than a random attachment is achieved. This also makes the coupler-matrix an affinity resin for only those DNA molecules having an end complementary to the exposed coupler restriction enzyme half-site. Third, the DNA molecule covalently immobilized through a restriction site can be released intact by restriction endonuclease cleavage.

Fourth, if the DNA molecule is asymmetric in its restriction enzyme half site ends, the DNA will attach to the coupler matrix by only the homologous end. This forces an orientation or alignment of all DNA molecules bound in the nucleic acid matrix, making opportunities for selectivity of reaction with the bound DNA. For instance, only one specific end will be labelled when subjected to polynucleotide kinase and γ- ^{32}P-ATP. This eliminates the need for subsequent strand separation, or restriction cleavage and subfragment purification when used for a restriction site mapping experiment. In fact, the restriction site mapping could be executed while the DNA remains immobilized.

Accordingly, it is an object of the present invention to provide a means for reversibly immobilizing a DNA molecule.

A further object of the invention is to provide a means for selectively attaching a DNA molecule to a solid support

through any desired terminal endonuclease restriction site.

Another object is to immobilize DNA in a selected orientation.

Still another object is to provide an affinity matrix for the separation of DNA molecules containing a specified terminal restriction site.

Various other objects of the invention will be apparent from the description above and from the description to follow of a preferred embodiment, taken in connection with the accompanying figures, in which:

FIGURE 1 shows the base pair sequence of a specific coupler according to the invention;

FIGURE 2 illustrates the preparation of that coupler;

FIGURE 3 illustrates one way of attaching the coupler to a matrix or solid support;

FIGURE 4 illustrates studies on the activation and verification of the coupler matrix;

FIGURE 5 illustrates the immobilization of DNA from plasmid pSP14 on the coupler matrix;

FIGURE 6 illustrates the reaction of various restriction endonucleases with the immobilized labelled pSP14 DNA;

FIGURE 7 illustrates the preparation of a second specific coupler and its base sequence; and

FIGURE 8 illustrates the binding of that coupler to DNA and the subsequent immobilization of the DNA-coupler system to a matrix.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention herein is illustrated by the following

description of the preferred embodiments. In one embodiment, a convenient coupler was isolated and attached to a solid support. In the second embodiment, a coupler was isolated and attached to DNA, and the resulting coupler nucleic acid system was immobilized on a solid support.

Example 1: Coupler I

In this example, a coupler was bound to a solid support. Then DNA was immobilized by attachment to the coupler matrix. The effectiveness of the coupler matrix in immobilizing a 3986 bp (base pair) plasmid DNA fragment was then studied.

The coupler used was a 182 bp double-strand DNA fragment, hereinafter called "Coupler I" from plasmid pUC9 (Vieira, J. and Messing, J., Gene 19 259 (1982)). It has six frequently used restriction endonuclease sites near its 5' end, as can be seen in FIGURE 1, which shows the base pair sequence in its entirety. FIGURE 2 illustrates the preparation of Coupler I. First, 1.5 mg of purified plasmid pUC9 was sequentially digested with restriction endonucleases Bgl I, then Hind III. Then the phenol extracted reaction products were subjected to NACS chromatography (Thompson, J. A., Blakesley, R. W., Doran, K., Hough, C. J. and Wells, R. D., Methods in Enzymology 100, 368 (1983)), first loading in 0.5 M NaCl, TE (10 mM Tris-HCl [pH 7.2], 1 mM Na_2 EDTA) buffer, then eluting with an increasing linear gradient (300 ml) 0.5-0.65 M NaCl in TE buffer. The purified 182 bp Coupler I was concentrated by ethanol precipitation, then resuspended in TE buffer. The progress of the Coupler I purification was monitored by subjecting samples to 1% agarose gel electrophoresis.

This is a convenient coupler because of its six restriction sites, but it will be clear to those skilled in the art that many other oligonucleotides may be isolated for the same purpose, using the methods disclosed in Thompson supra, which disclosure is incorporated herein by reference. Others, of course, can be synthesized "to order", using the methods

disclosed in Narang, S. A., Hsiung, H. M. and Brousseau, R., Methods in Enzymology 68, 90 (1979), which disclosure is incorporated herein by reference.

A ribonucleotide was then added to the 3'-OH end of the Bgl I half-site of Coupler I, then periodate oxidized and linked to hydrazide cellulose. The specific process is shown in FIGURE 3. A 2.0 μg aliquot of the purified 182 bp Coupler I was incubated with 16 units of terminal deoxynucleotidyl transferase (TdT) and 96 μM rATP, selectively introducing a short oligoribonucleotide to the 3' extended Bgl I terminus. The resulting adenylylated Coupler I was oxidized by treatment with 1.0 mg of $NaIO_4$ for one hour at 4°C. This converted the ribose cis-diol of the 3'-terminal ribonucleotide to a dialdehyde (rA*). When mixed with 0.01 gm of cellulose (or agarose) adipic acid dihydrazide matrix, Coupler I reacted, became covalently attached and oriented as shown.

There are of course other ways to attach such an oligonucleotide covalently to a solid support. For example, the coupler could be ligated by RNA ligase to a pAp already coupled to cellulose. The coupler can be modified by various chemical methods at its 3' or 5' ends to allow covalent attachments to a solid support (Chu, B. C. F., Wahl, G. M., Orgel, L.E., Nucleic Acids Res. 11, 6513 (1983); Shabarora, Z. A., Ivanovskaya, M. G., and Isaguliants, M. G., FEBS Lett. 154, 288 (1982); Shih supra and Potuzak supra.). Still other appropriate supports and binding methods will be apparent to those skilled in the art. It may be convenient to attach the coupler to the solid support by means of some intermediate molecule or molecules. Our invention comprehends the existence of any such intermediates, so long as the coupler is bound to the solid support by bonds which are not disturbed by subsequent DNA manipulations.

A variety of solid supports may be employed. In general the term "solid support", as used herein, refers to any of the water-insoluble matrices described in U.S. Letters Patent

No. 4,342,833 to Chirikjian, column 3, lines 15-58.

Table I shows the results of studies on the efficiency of Coupler I attachment, as compared to attachment of tRNA by the same process.

TABLE I

EFFICIENCY OF LINKER ATTACHMENT

SUBSTRATE	AMOUNT SUB-STRATE (CPM $_6$ X 10^{-6}	AMOUNT BOUND (CPM $_6$ X 10^{-6})	AMOUNT NOT BOUND (CPM $_6$ X 10^{-6})	ATTACH-MENT EFFI-CIENCY (PERCENT)
tRNA	4.42	0.07	4.31	1.6
$tRNA_{OX}$	4.98	4.26	0.72	85.5
182 bp LINKER	5.32	0.06	5.21	1.1
182 bp $LINKER_{OX}$	4.71	1.76	2.92	37.4*

*Uncorrected for terminal deoxynucleotide transferase (TdT) reactivity; when corrected, this value becomes 85.5.

A volume of either tRNA or the 182 bp Coupler I (adenylylated with TdT and rATP), each ^{32}P-labeled at the 5' end with T4 polynucleotide kinase and γ-^{32}P-ATP, was divided into equal aliquots. One aliquot was subjected to periodate oxidation (OX), the other aliquot left untreated, then both aliquots reacted with the hydrazide matrix. After incubation, the entire volume of reaction was quantitated by Cherenkov radiation detection (amount of substrate). The reaction mixture was then filtered through a small column containing glass wool. The filtrate (amount not bound) as well as the insoluble matrix trapped by the filtration (amount bound) were individually quantitated as above. Attachment efficiency was calculated as the ratio of the amount bound to the amount of substrate times 100%. These

values represent averages of duplicate experiments. From these data it can be seen that the Coupler I attachment efficiency, that is, percentage of the substrate bound to the matrix, was 85.5.

Further studies on the coupler-matrix combination, hereinafter "Coupler I Matrix," were performed as shown in Table II, and in FIGURES 4 and 5. FIGURE 4 illustrates an experiment to examine the orientation and suitability of the prepared Coupler I Matrix for reaction. Aliquots of labelled Coupler I Matrix were treated by various techniques, as follows, the results of which are seen in Table II.

TABLE II

SUITABILITY OF LINKER MATRIX FOR REACTION

TREATMENT	AMOUNT SUB-STRATE ($CPM_4 X 10^{-4}$)	AMOUNT BOUND ($CPM_4 X 10^{-4}$)	AMOUNT RE-LEASED ($CPM X 10^{-4}$)	PER-CENT RE-LEASED
ENZYME REACTION				
BUFFER ONLY	4.85	4.81	0.02	0.5
BUFFER PLUS				
EcoR I	4.92	0.99	3.89	79
BamH I	5.31	1.51	3.77	71
Pst I	4.86	1.28	3.55	73
BAP	5.06	1.20	3.85	76
DENATURATION				
90% FORMAMIDE	5.16	4.05	1.08	21
7 M Urea	4.83	4.06	0.82	17
90°C	4.79	3.55	1.25	26

The indicated amount of substrate (5'-end ^{32}P- labeled Coupler I Matrix) was placed in a microfuge tube, rinsed several times with buffer (50 mM Tris-HCl [pH 8], 50 mM NaCl and 10 mM $MgCl_2$), then incubated at 37°C for 3 hours as is or after the addition of 40, 40, 40 or 3,000 units EcoR I, BamH I, Pst I, or

bacterial alkaline phosphatase (BAP), respectively. The reactions were individually filtered through a column of glass wool. The filtrate (amount released), the trapped matrix (amount bound) as well as the amount of substrate were quantitated by Cherenkov radiation detection. The percent released was determined as the ratio of the amount released to the amount of substrate times 100%. These values represent averages of duplicate experiments.

To the indicated amount of substrate (Coupler I Matrix) in a microfuge tube was added 1 ml of either 90% formamide, 7 M urea or buffer (20 mM Tris-HCl [pH 7.2], 1 mM Na_2 EDTA). The tubes were incubated for 10 minutes at either 37°, 37° or 90°C, respectively. After treatment, the reaction volumes were filtered and quantitated as described in the preceding paragraph.

The restriction endonuclease digestion products in the soluble volume were characterized by subjecting samples of each to electrophoresis in a 12% polyacrylamide gel, followed by autoradiography.

In the final tests, DNA from plasmid pSP14 (Anderson, S., Gait, M.H., Magal, L. and I.G. Young, Nucleic Acids Res. 8, 198 (1980)) was immobilized on the Coupler I Matrix and then manipulated. FIGURE 5 shows the immobilization process, which used phage T4 DNA ligase to bind an EcoR/BamH I restriction fragment to Coupler I Matrix.

A 1.0 mg aliquot of NACS purified plasmid pSP14 was incubated with Eco R I, followed by BamH I, then purified from a 26 bp cleavage fragment by NACS chromatography. The 3986 bp pSP14 fragment, concentrated by ethanol precipitation, was further incubated with BAP. Approximately 100 µg (40 pmole) of this pSP14 fragment was mixed with Coupler I Matrix (about 50 pmole of bound Coupler I) which had been activated by EcoR I digestion as in FIGURE 4, and 20 units of T4 DNA ligase. After 2 hours at 22°C, the ligated pSP14 Coupler I Matrix was filtered

through sintered glass. Measurement of the absorbance at 260nm (A_{260}) of the filtrate indicated that approximately 75% of the pSP14 input A_{260} was immobilized.

FIGURE 6 illustrates studies involving labelling, endonuclease reaction, and reaction products of the immobilized pSP14 DNA. As seen in FIGURE 6, approximately 50 µg of immobilized pSP14 was 5'-end ^{32}P labelled by incubation with 55 units of T4 polynucleotide kinase and 0.5 mCi γ-^{32}P-ATP for one hour at 37°C. The matrix was extensively washed with 2 M NaCl in TE buffer followed by water.

The matrix volume was divided into five aliquots, then each separately incubated with either EcoR I, Pst I, Pvu II, Ava I or buffer alone. The reaction products were identified by separation with 1% agarose gel electrophoresis, followed by audioradiography. Only single fragments of sizes 3896, 3236, 1692 and 1049 base pairs respectively, were found in the eluate. This corresponds to the sizes expected if pSP14 were attached to the Coupler I Matrix through only the Eco RI end.

Example 2: Coupler II

In this example, a DNA was bound to an activated coupler. The coupler nucleic acid system was immobilized by attachment to a solid support.

The coupler used was a tetradeoxynucleotide, hereinafter called "Coupler II". The sequence of the coupler is 5'-d(TpCpGpG)-3' as seen in FIGURE 7, and this sequence is complementary to one of the half-site sequences generated by digesting DNA with the restriction enzyme AvaI. FIGURE 7 illustrates the activation of the coupler. First, 33 mg of the coupler was ^{32}P-labeled at the 5' end with T4 polynucleotide kinase and γ-^{32}P-ATP. Unincorporated γ-^{32}-ATP was removed by NACS chromatography.

The ^{32}P- labeled coupler was incubated with 300 units of terminal deoxynucleotidyl transferase (TdT) and 96 µM rATP, selectively introducing an oligoribonucleotide to the 3'- end of

the coupler. Following purification by NACS chromatography, the resulting adenylylated coupler was oxidized by treatment with 1.0 mg of $NaIO_4$ for one hour at 4°C. This converted the ribose cis-diol of the 3'- terminal ribonucleotide to a dialdehyde (rA*). When mixed with 1.0 mg of biotin hydrazide (BH), the coupler reacted, become covalently attached and activated in the orientation shown, hereinafter called "Activated Coupler II".

FIGURE 8 illustrates the method whereby Activated Coupler II is bound to a DNA molecule to generate a coupler-nucleic acid system which can be subsequently immobilized to a solid support. A double stranded DNA restriction fragment containing asymmetric ends, only one of which was complementary to Coupler II, was incubated with BAP to remove the 5'- terminal phosphate and prevent self ligation of this DNA. Approximately 100 μg (140 pmole) of this DNA was mixed with 3.0 μg (1713 pmole) of the ^{32}P- labeled activated coupler and 100 units of T4 DNA ligase. After two hours at 22°C, the resulting coupler-nucleic acid system was purified away from the unreacted activated coupler using NACS chromatography. A_{260} measurements and radioactive counting of the purified coupler nucleic acid system indicated that greater than 90% of the DNA had been linked to the activated coupler.

FIGURE 8 further illustrates that the coupler-nucleic acid system was immobilized by attachment to a solid support utilizing the specific high affinity between biotin and streptavidin (Green, N.M., Advances in Protein Chemistry, 29, pp. 85-133, 1975). A 10 mg aliquot of the coupler-nucleic acid system was mixed with 0.49mg of streptavidin covalently attached to agarose (solid support) in 20 mM Tris-HCl (pH 7.2), 0.1 ml of 1mM Na_2 EDTA. After 5 minutes at 22°C, the mixture was filtered through glass wool. A_{260} measurements and radioactive counting determined that 92% of the coupler-nucleic acid system was immobilized by this procedure.

The invention has been described with particular emphasis on the preferred embodiments, but it should be understood that variations and modifications within the spirit and scope of the invention may occur to those skilled in the art to which the invention pertains.

We claim:

1. A nucleic acid-matrix system comprising:

a solid support;

a coupler including a DNA molecule having first and second ends, the first end being covalently bound to the solid support and the second end being unbound to the solid support and being a restriction enzyme half site; and

a DNA molecule having a first end with a restriction half site complementary to the second end of said coupler, said molecule being coupled at said complementary half site to said second end of said coupler.

2. The invention of Claim 1 in which said coupler comprises a restriction fragment isolated from naturally occurring DNA by sequential treatment of said DNA with two different restriction endonucleases.

3. The invention of Claim 2 in which said naturally occurring DNA is obtained from the DNA of plasmid pUC9.

4. The invention of Claim 3 in which said two different restriction endonucleases are Bgl I and Hind III.

5. The invention of Claim 1 in which said coupler comprises oligonucleotide and mononucleotide, said mononucleotide having been chemically bonded to said oligonucleotide in vitro.

6. The invention of Claim 5 in which said oligonucleotide is a synthetic single strand having the base sequence 5'-d(TpCpGpG)-3'.

7. The invention of Claim 6 in which said oligonucleotide has had added to its 3'-OH end a biotinylated ribonucleotide.

8. The invention of Claim 7 in which said solid support is agarose to which streptavidin is covalently attached, and said biotinylated ribonucleotide has been bonded by condensation to said streptavidin.

9. The invention of Claim 5 in which said oligonucleotide includes a ribonucleotide attached to its 3'-OH end.

10. The invention of Claim 9 in which said solid support is cellulose to which adipic acid hydrazide is covalently attached, and said ribonucleotide has been converted to a dialdehyde by periodate oxidation, and said dialdehyde has been bonded by condensation to said adipic acid hydrazide.

11. The invention of Claim 1 in which said solid support is cellulose to which adipic acid hydrazide is covalently attached.

12. The invention of Claim 1 in which said coupler comprises a restriction fragment and a mononucleotide, said mononucleotide having been chemically bonded to said restriction fragment in vitro.

13. The invention of Claim 12 in which said restriction fragment includes a ribonucleotide attached to its 3'-OH end.

14. The invention of Claim 13 in which said solid support is cellulose to which adipic acid hydrazide is covalently attached, and said ribonucleotide has been converted to a dialdehyde by periodate oxidation, and said dialdehyde has been bonded by condensation to said adipic acid hydrazide.

15. The invention of Claim 1 in which said solid support is agarose to which streptavidin is covalently attached.

16. A coupler matrix system comprising:

a solid support; and

a coupler having an end covalently bound to the solid support and a section extending from said bound end and containing a restriction enzyme recognition site;

wherein said recognition site can be activated to convert said recognition site to a restriction enzyme half site.

17. The invention of Claim 16 in which said coupler comprises a restriction fragment isolated from naturally occurring DNA by sequential treatment of said DNA with two different restriction endonucleases.

18. The invention of Claim 16 in which said coupler comprises a restriction fragment and a mononucleotide, said mononucleotide having been chemically bonded to said restriction fragment in vitro.

19. The invention of Claim 16 in which said solid support is cellulose to which adipic acid hydrazide is covalently attached.

20. The invention of Claim 17 in which said naturally occurring DNA is obtained from the DNA of plasmid pUC9.

21. The invention of Claim 20 in which said two different restriction endonucleases are Bgl I and Hind III.

22. The invention of Claim 18 in which said restriction fragment has had added to its 3'-OH end a ribonucleotide.

23. The invention of Claim 22 in which said solid support is cellulose to which adipic acid hydrazide is covalently attached, and said ribonucleotide has been converted to a dialdehyde by periodate oxidation, and said dialdehyde has been bonded by condensation to said adipic acid hydrazide.

24. A coupler nucleic acid system comprising:

a DNA molecule having a first end with a restriction enzyme half site; and

a coupler including a second DNA molecule having a first end with a restriction enzyme half site complementary to the restriction half site of the first DNA molecule, said first end being coupled to said end of the first DNA molecule, and a second end connectable to a solid support.

25. The invention of Claim 24 in which said coupler comprises an oligonucleotide to which biotin has been added.

26. The invention of Claim 24 in which said DNA is a restriction endonuclease fragment.

27. The invention of Claim 25 and in which said oligonucleotide is a single strand having the base sequence 5'-d(TpCpGpG)-3'.

28. The invention of Claim 26 in which said restriction endonuclease is AvaI.

29. A process for attaching an exposed half-site of a restriction site for a given restriction endonuclease to a solid support, said process comprising, in any order,

binding covalently to said solid support a coupler including an oligonucleotide containing said restriction site; and

cleaving said oligonucleotide at said restriction site with said restriction endonuclease to produce said exposed half-site.

30. The process of Claim 29 in which said solid support is a matrix of cellulose or agarose adipic acid dehydrazide, and the said chemical bonding of the Bgl I end of said restriction fragment to said solid support is achieved by

adding a ribonucleotide to the end of the Bgl I half-site of said restriction fragment;

oxidizing said ribonucleotide with periodate to convert its ribose cis-diol to a dialdehyde; and

mixing said restriction fragment with said matrix.

31. A process for attaching an exposed half-site of a restriction site for any desired one of the restriction endonucleases Hind III, Pst I, Hinc II, BamH I, Sma I or EcoR I to a solid support, said process comprising isolating from plasmid pUC9 DNA a Bgl I/Hind III restriction fragment;

chemically bonding the Bgl I end of said restriction fragment to said solid support; and

cleaving said restriction fragment with the said restriction endonuclease for which an exposed half-site is desired.

32. A process for immobilizing DNA on a solid support, said process comprising

binding stably to said solid support by one end an oligonucleotide having on its other end a first half-site for a

known restriction endonuclease, and for which said DNA contains a recognition site for said restriction endonuclease;

cleaving said restriction site on said DNA with said restriction endonuclease to form second and third half-sites on said DNA; and

joining said first half-site to either of said second and third half-sites by treatment with DNA ligase.

33. A process for immobilizing DNA on a solid support, said process comprising

binding said DNA to an oligonucleotide coupler through corresponding restriction endonuclease half-sites located on an end of said DNA and a first end of said coupler; and

binding said coupler stably to said solid support by a second end of said coupler.

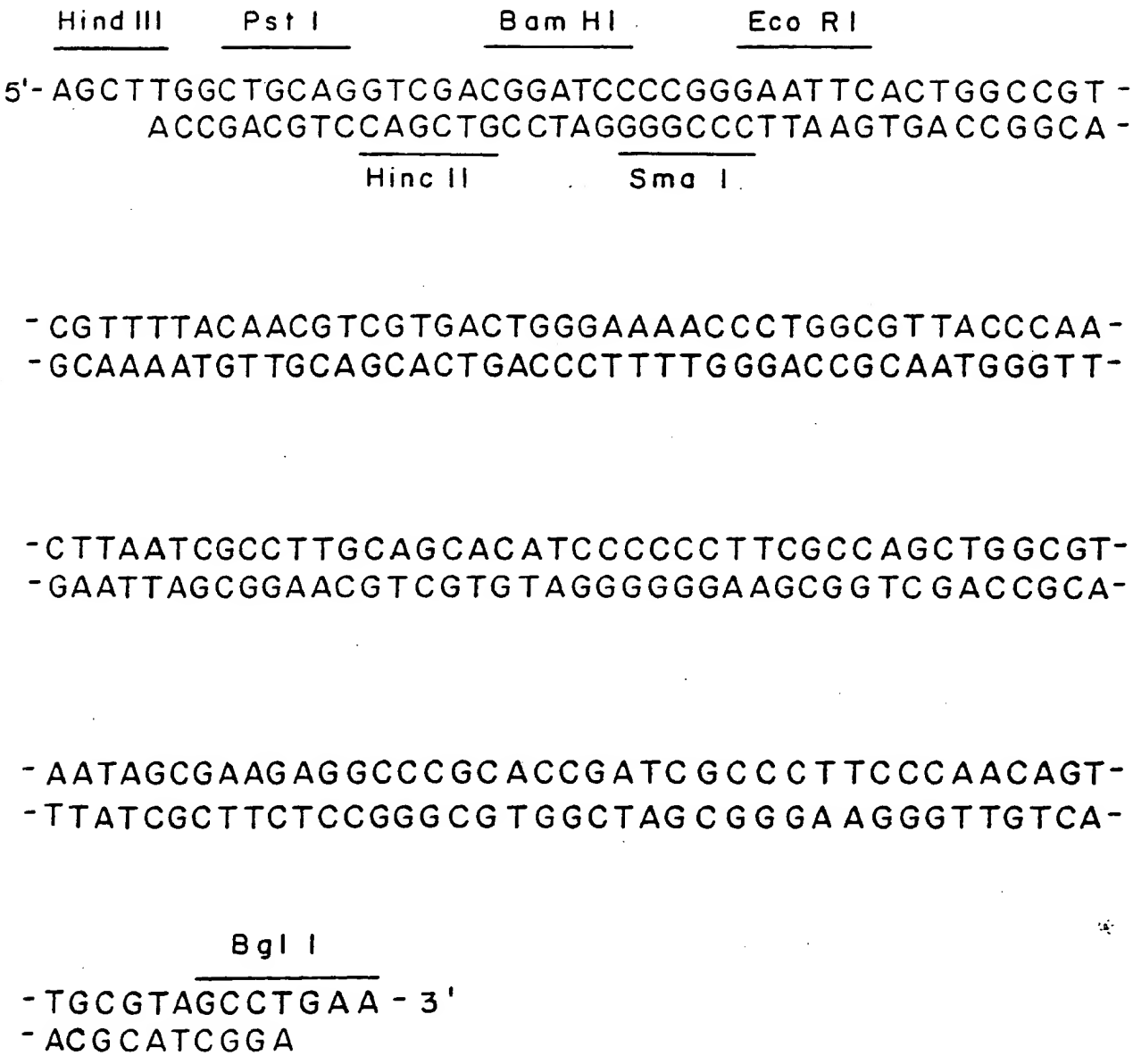

FIGURE 1

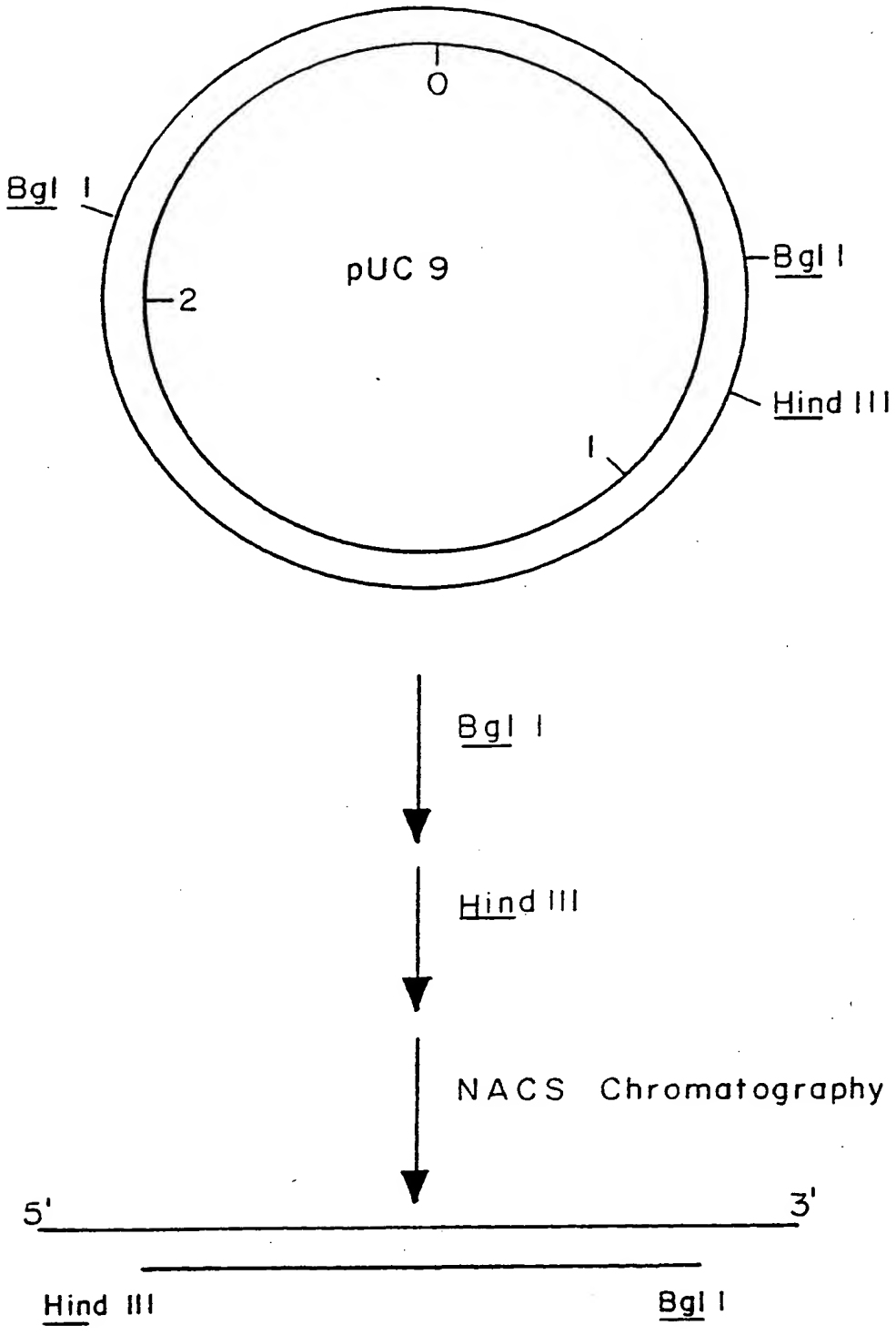

FIGURE 2

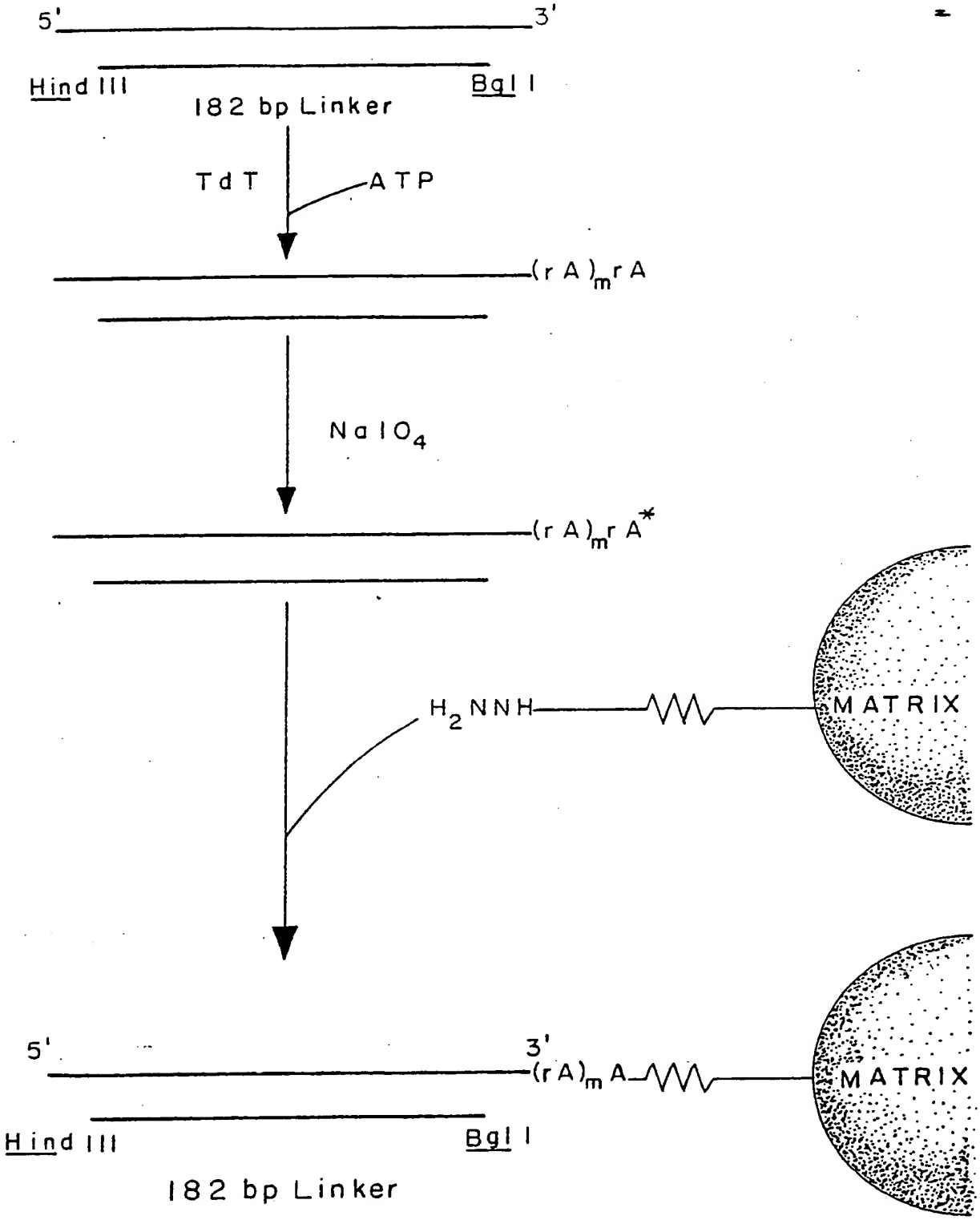

FIGURE 3

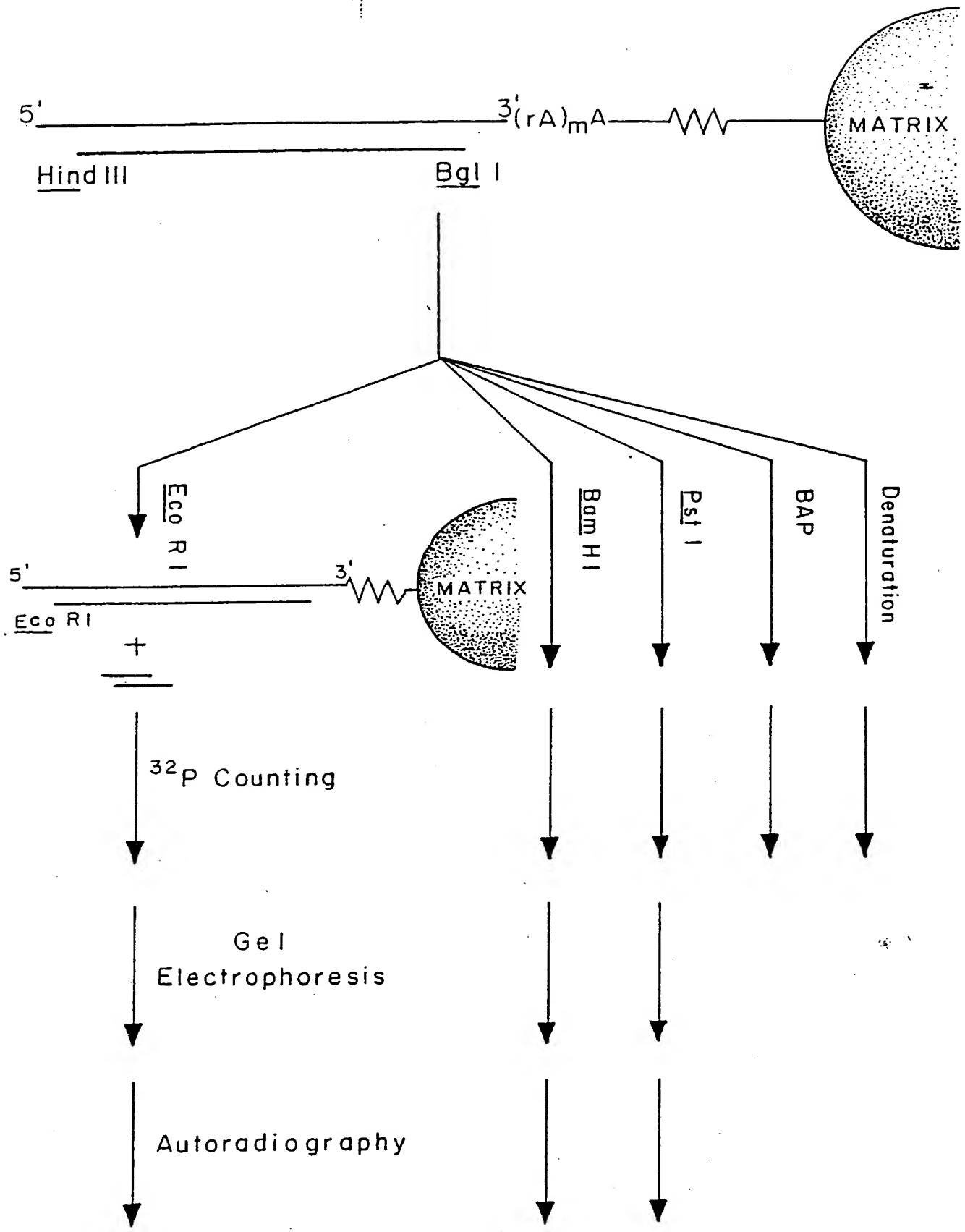

FIGURE 4

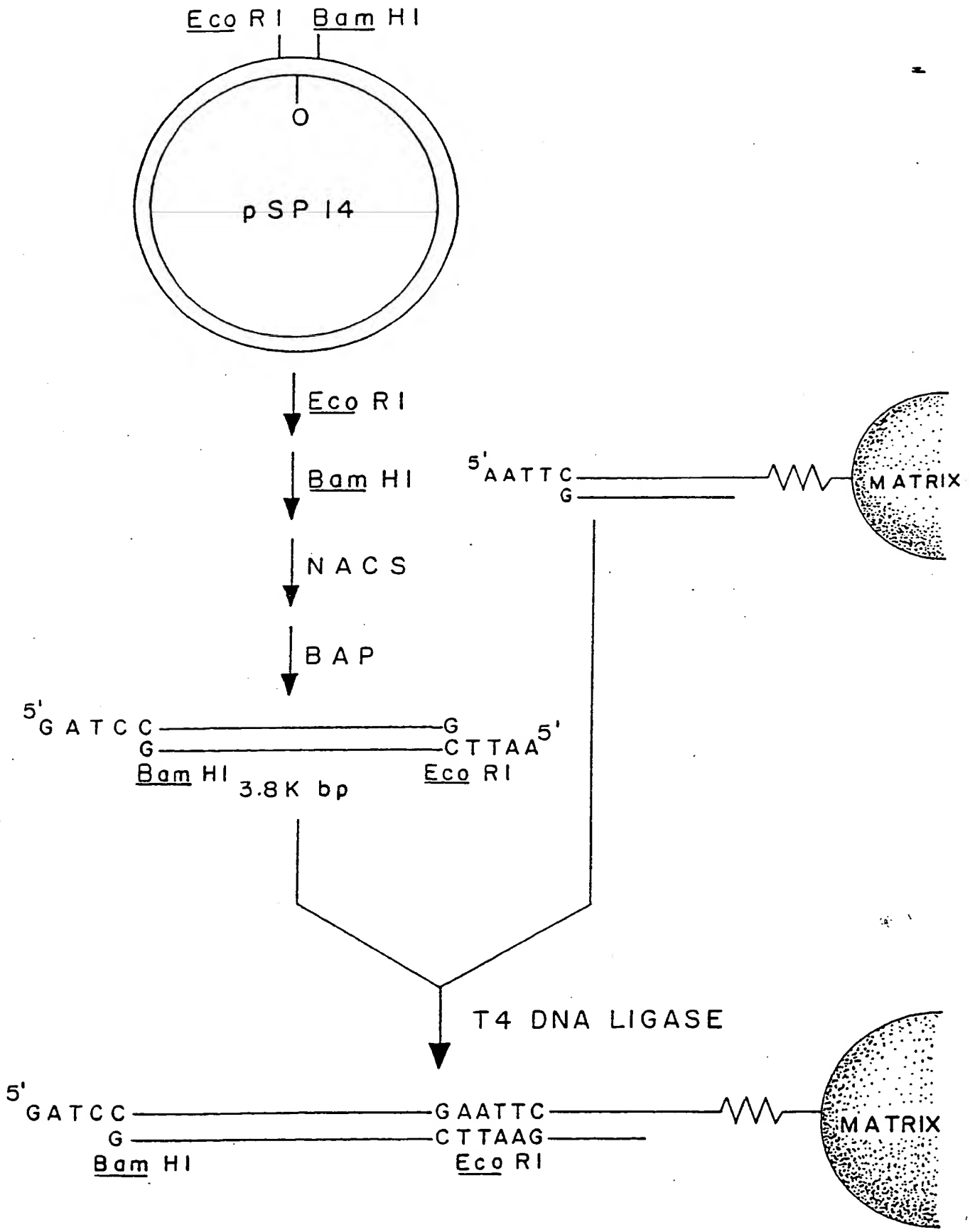

FIGURE 5

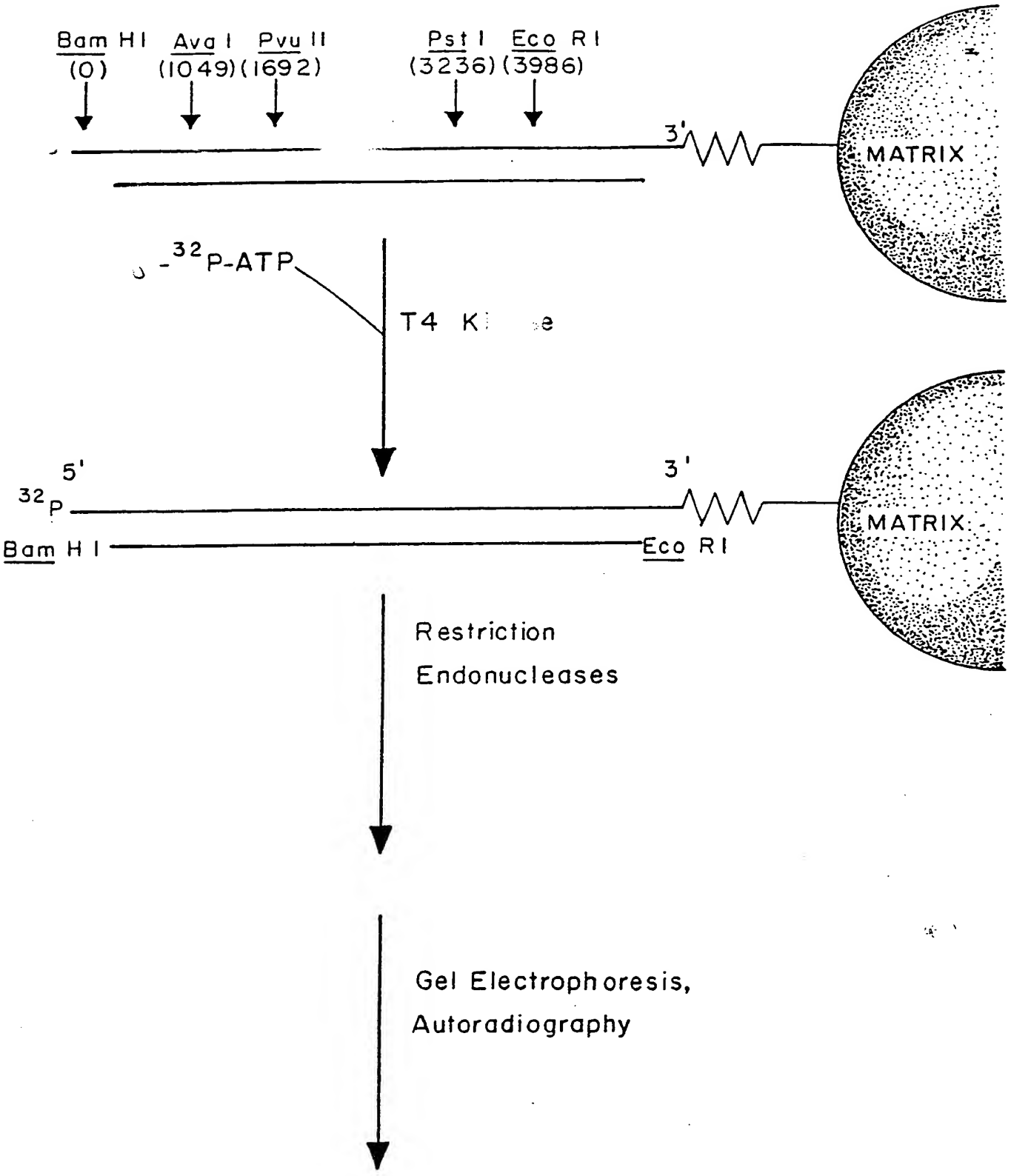

FIGURE 6

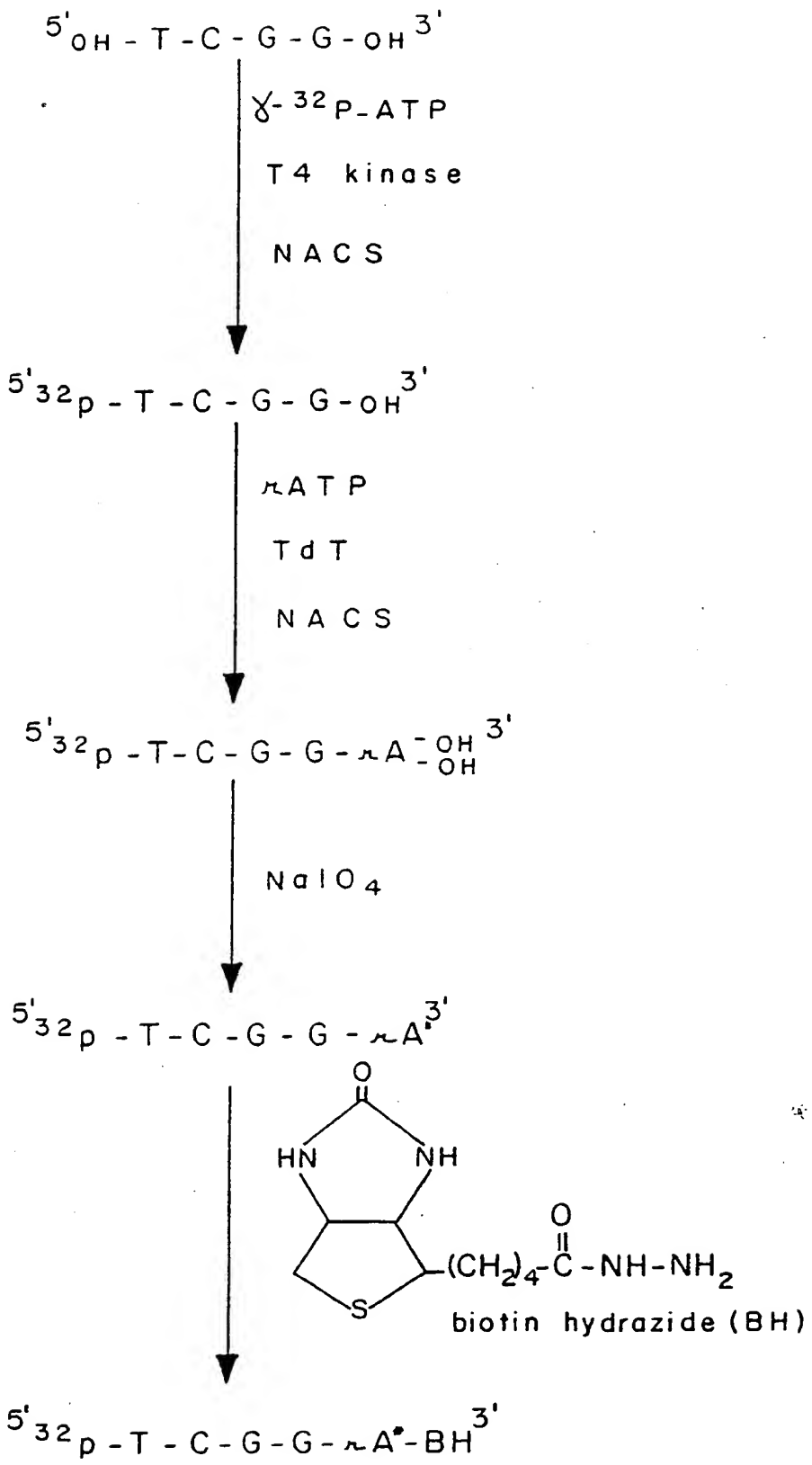

FIGURE 7

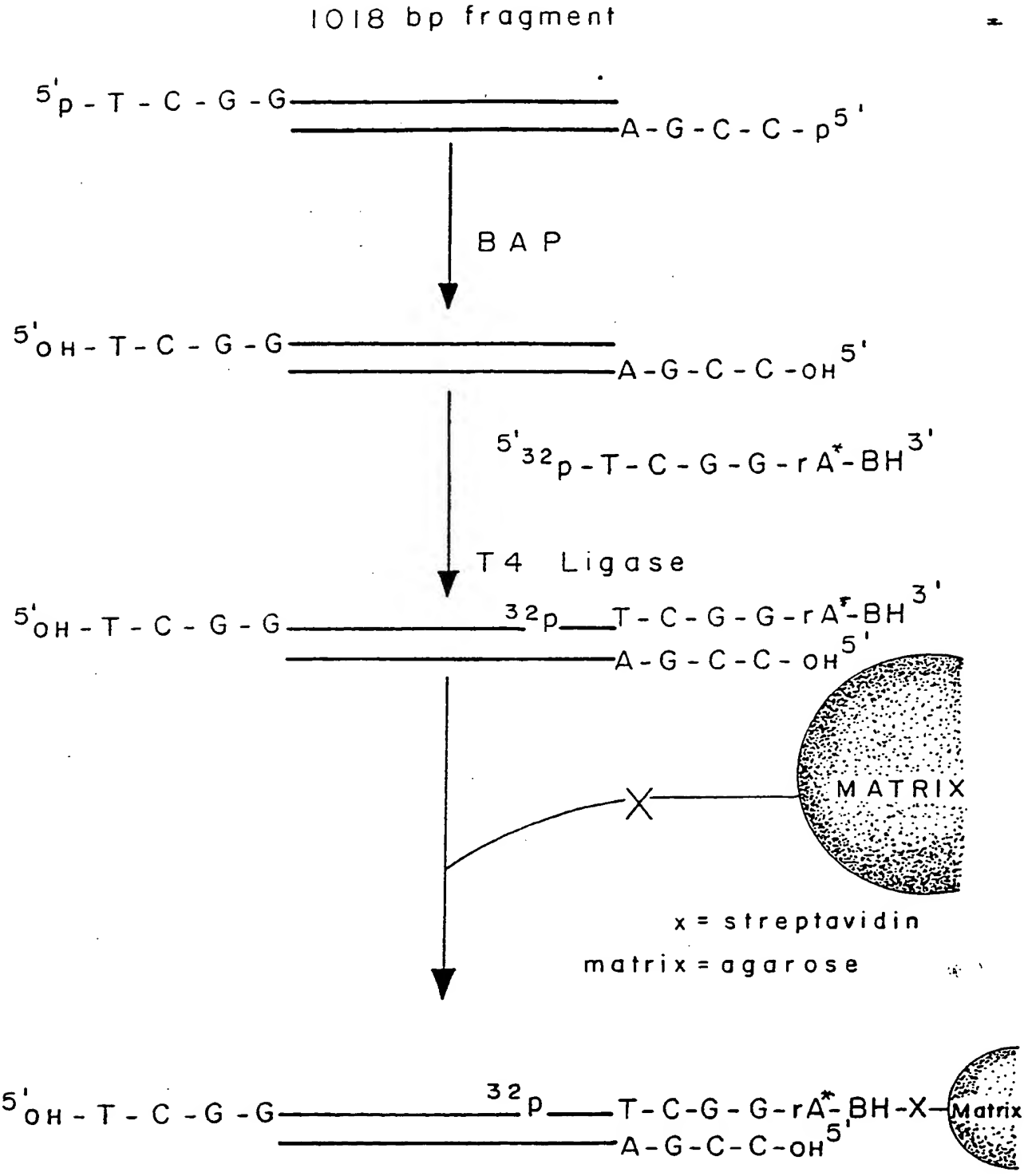

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/00508

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

US: 435/91

IPC: C12P 19/34

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	422/56, 58; 435/6, 91, 172(for.); 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts 1967 - 1984

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,321,365, published 23 March 1982, WU et al.	1,2,4,5,9, 12,13,16,24, 26,29,32,33
Y	EP, A, 0035384, published 09 September 1981, Rutter	1,5,9,16, 24,26
A	N, Gross et al., Journal of Biological Chem. Vol. 257, No. 9 issued 10 May 1982, pages 4738-4745.	
A	US, A, 4,342,833, published 03 August 1982, Chirikjian	4,21,27,28, 30,31
A	WO, A, 8303363, published 13 October 1983, Reisner et al.	
A	US, A, 4,139,346, published 13 February 1979 Rabbani	

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
22 June 1984	03 JUL 1984
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	J. Peter Fasse